

STUDIES ON SECONDARY TETANUS IMMUNIZATION IN MICE

I. Dose-Response Relationship¹

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In a previous study on secondary diphtheria-pertussis-tetanus combined vaccination in children (1), it was demonstrated that over the range of initial antibody titer studied, a rectilinear relationship existed between the log prevaccination titer and the log fold-increase in titer after booster injection. Small increase in titer was correlated with high prevaccination titer, and *vice versa*. The dose-response slopes of 0.5 ml series of the three systems were around -0.50, and decreasing the booster dose from 0.5 to 0.2 ml resulted in slightly steeper slopes. On reviewing the literature, similar response patterns were found to exist in secondary immunization of diphtheria, tetanus, epidemic typhus, virus influenza and poliomyelitis in human-beings (2, 3, 4, 5, 6).

Tetanus toxoid as a secondary antigenic stimulus is particularly suitable for studying various phase of immune reactions, because of its well-known capacity to yield a typical secondary immune response. As a starting point for this series of studies of secondary tetanus immunization in mice, the dose-response relationship was first investigated to see whether the above-mentioned generalization of secondary immune response patterns in men holds true in this species. The intensity of booster stimuli employed in this study was purposely spaced at wide intervals of 1 log, in order to bring out more clearly the response patterns under the influence of widely different doses.

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MATERIALS AND METHODS

Mice

200 female mice of strain *dd* weighing 16-18 g were used. Groups of 50 animals were raised separately in 4 metal cages and fed with grain mixture, and tap water *ad libitum*. During the course of immunization 4 mice escaped, 8 were discarded for unsatisfactory weight gain or unhealthy appearance, and from the remaining 188 mice 186 duplicate blood samples were obtained for antitoxin titration before and after booster injection. The mice weighed 24-29 g at the time of the booster.

Toxoid and Immunization

The plain tetanus toxoid used was the product of ammonium sulphate purification with a purity of 1234 Lf per mg of trichloroacetic acid precipitable nitrogen. In order to obtain widespread residual immunity at the subsequent booster immunization, 4 groups of mice were given, subcutaneously, 2 primary immunizing doses of different strengths of toxoid, 12 days apart. 3 months after the second injection, 4 new booster groups were formed by redistribution of the animals under test: each original group was divided into 4 subgroups of approximately equal numbers, and each contributed 1 subgroup to form 4 new groupings which received subcutaneously different booster doses of tetanus toxoid at 1 log intervals. A schematic presentation of the experimental plan follows below for the purpose of clarity:

Group	Primary Immuniz.		Secondary Immuniz.
	Lf	Mice redistributed to form new booster groups	Lf
1	3	3	0.5
2	1	1	0.05
3	0.3	0.3	0.005
4	0.3	0.1	0.0005

Plasma samples were kept at -20°C before titration. Chen's method of titrating low-titered tetanus antitoxin in small samples (8-9) was used in this study.

RESULTS

The results are recorded in detail in Table I. It will be noted that the terms "titer increase" or "increase in titer", used interchangeably throughout this paper, are expressed as fold-increase in titer after secondary immunization.

The pertinent data on 0.0005 Lf series are omitted from Table I, because titration of this series was discontinued, on the grounds that earlier titrations indicated that the minute dose of 0.0005 Lf was apparently too weak to serve as booster stimulus under the conditions of the experiment. In this series, only 3 out of 20 mice tested exhibited barely measurable titer increase of 2-4 fold after secondary immunization. Furthermore, such feeble increase in titer was not correlated with any particular range of pre-booster titer. Since these feeble reactions could be interpreted as the results of experimental errors and/or fluctuation of individual response

Bleeding and Antitoxin Titration

Duplicate blood samples were collected from each mouse 8-10 days before, and 9 days after the booster injection. The first sample was obtained by puncturing the jugular vein after the method of Kassel and Levitan (7), and the second by decapitation. 0.4-0.6 ml of blood was collected with a syringe or a calibrated tube which contained 0.2 ml of citrate anticoagulant, and after thorough mixing the citrated blood was subjected to a centrifugal force of 400 *g* for 10 minutes. From the measured volume of citrated blood, the dilution factor of each plasma sample was computed on the assumption that there were equal volumes of cellular components and plasma in the blood, and corrections were made thereby for interpretation of titration results.

TABLE I
Titer increase in circulating tetanus antitoxin in mice following revaccination with varying doses of tetanus toxoid

Booster Dose, Lf	Pre-booster Titer, u/ml												Grand Total
	<.001	.001	.002	.004	.008	.016	.032	.064	.128	.256	.512	1.024	
0.5	1 3 512 1	32 1 64 1 128 1 1024 1		2 1	64 1	128 1 256 1 1024 1	2 1 128 3 256 1	4 1 64 2 128 2	8 1 16 1 32 1 64 2 256 1 512 1	2 1 8 1 16 1 32 1 64 1	16 2 32 1 64 1	2 8 16 1 32 1 64 1	45
Total	2	4		1	1	3	5	5	7	8	6	3	
0.05	1 3 16 1 512 1	1 3 256 2 512 1	16 1 64 1 128 1	2 1	512 1	8 1 32 2 128 1	64 1 128 1	32 1 64 1 128 1	1 1 8 1 16 1 32 4 64 2 256 1	16 2 32 1 128 1	4 4 8 2 16 1	2 2 8 2	47
Total	5	6	9	1	1	4	2	3	10	4	4	4	
0.005	1 2 8 1 1024 1	1 4 8 1 1024 1	1 1 512 1	16 3	512 1	1 1 4 1 8 2 128 1	8 2 16 1 64 1	4 1 8 2 16 2 32 3	2 2 4 2 8 2 16 2 32 1	1 1 2 2 4 1	1 1 2 2	1 1 2 2	49
Total	2	6	1	1	3	5	4	8	9	4	3	3	
Total sum	9	16	4	3	5	12	11	16	26	16	13	10	141

Arabic figures denote fold-increase in titer, italic figures case number.

TABLE II

Geometrical mean titer increase of circulating tetanus antitoxin in mice following secondary immunization

Booster Dose Lf	Pre-booster Titer, u/ml				
	Titer Range	<0.001-0.002	0.004-0.016	0.032-0.128	0.256-1.024
0.5		71.8 (8.77) <i>4.6</i>	84.5 (7.97) <i>2.6</i>	56.6 (4.35) <i>1.5</i>	24.0 (2.54) <i>2.4</i>
0.05		15.3(12.91) <i>5.7</i>	32.0 (5.99) <i>2.0</i>	36.8 (3.57) <i>3.3</i>	10.1 (2.97) <i>7.2</i>
0.005		2.7 (8.91)	16.0 (5.64)	11.1 (2.65)	1.4 (1.58)

Arabic figures denote mean titer increase, those within the parenthesis standard deviations, both geometric. Italic figures denote ratios between the upper and lower means.

after all, and the elucidation of either cause is beyond the scope of the present study, it was decided that 0.0005 Lf of the toxoid used in this study was inadequate for booster stimulation, so further titration of the remaining plasma samples was discontinued.

Table I shows that the total observation number of the 3 dose groups is comparable. Generally speaking, the experimental plan appears satisfactory in that the primary immunization schedule yielded, in all 3 series, a range of residual immunity the extremes of which differ more than 1000-fold at the time of secondary immunization. However, the distribution of cases preponderates slightly to the high initial titer side, the unbalance being most apparent at the range of 0.002-0.008 u/ml plasma where truncation of the experimental data threatens. Critical analysis of the response on the basis of the original data is rendered all the more difficult by the following considerations: the case number at a single immunity level is generally small, and the variation of titer increase at each level is relatively big. Accordingly, the figures of titer increase in each series are assembled at intervals of 0.9 log (8-fold), and the geometrical means of their titer increase and related standard deviations calculated. The figures thus obtained are given in Table II, and a graphic presentation of the geometrical mean titer increase plotted against pre-booster antitoxin titer on a log-log scale is shown in Fig. 1. A value of 0.0005 is assigned arbitrarily to the class <0.001 u/ml for the purpose of computation.

The data in Table II and Fig. 1. indicate that at any pre-existing immunity level, a bigger booster dose invariably induces a greater titer increase. However, the extent of difference of dose-response varies with the level of pre-existing immunity, as shown by the ratio figures in

Table II. Thus the ratios of mean titer increase between graded doses obtained at the extremes of pre-existing titer level are about twice as great as those at the intermediate range, indicating that in secondary immunization, the influence of dose variation on titer increase is more pronounced in those mice with relatively high or low residual immunity. This is particularly true in those mice with low pre-booster titer range of <0.001-0.002 u/ml plasma, in which the ratio figures of 4.6 and 5.7 are consistently about twice as large as the respective figures of 2.6, 1.5 and 2.0, 3.3 in those with intermediate initial titer range of 0.004-0.128 u/ml. In mice with high pre-booster titer range of 0.256-1.024 u/ml, on the other hand, the greater ratio of mean titer increase between graded doses is not so constant. In this case the ratio figure of 7.2 between 0.05

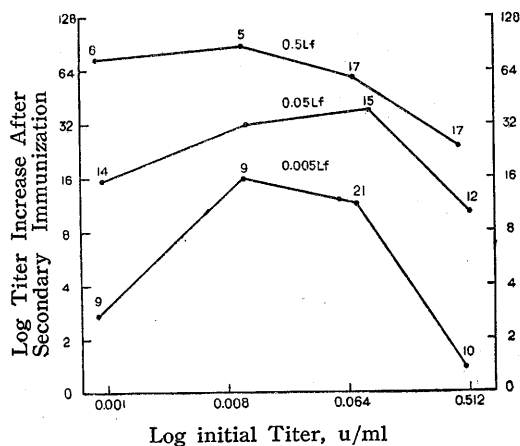


Fig. 1. Dose-response relationship in secondary tetanus immunization in mice. ● denotes the geometrical mean derived from a sample size indicated by figures above the points.

and 0.005 Lf remains about 2-3 times greater than the corresponding figures of 2.0 and 3.3 in the intermediate range, but the ratio figure of 2.4 between 0.5 and 0.05 Lf is not significantly greater than the corresponding figures of 2.6 and 1.5. In all probability the relatively small ratio of 2.4 between 0.5 and 0.05 Lf represents mainly the result of the inability of 0.5 Lf to incite a greater mean titer increase than 24-fold, conditioned by the ceiling phenomenon (2) under specified experimental factors. Whatever the underlying mechanism may be, the overall experimental data do not contradict the general picture of greater influence of dose variation upon titer increase in the high initial titer range than in the intermediate titer range. Thus by pooling the ratio figures of 2.4 and 7.2 in the range of high pre-booster titer an average of 4.8 is obtained, which is exactly twice as big as the corresponding averages of 2.3 and 2.4, when the ratio figures in the intermediate ranges of 0.004-0.016 and 0.032-0.128 u/ml plasma are similarly treated.

So far as the titer increase after secondary tetanus immunization is concerned, the reason that mice with relatively high or low level of pre-existing immunity are more sensitive to dose variation, than those with intermediate range of residual immunity, is made clear by inspection of Fig 1. Along the base the existence of a non-response line, given by a weak dose such as 0.0005 Lf, may be postulated. A slightly stronger threshold booster dose results in a response parabola, with the greatest titer increase around the intermediate range of pre-booster titer 0.008-0.064 u/ml plasma, comparatively smaller increase in the lower titer range, and least increase in the higher range. As the booster dose increases in intensity, these basic characteristics of different magnitudes of response at various levels of pre-existing immunity remain essentially unaltered other than quantitative modification in minor aspects: the titer increase at the extremes of pre-existing immunity level, the lower side in particular, accelerates at a great speed so that the steepness of the response parabola diminishes steadily. As a result the response curve of 0.5 Lf, the greatest dose employed in this study, looks almost, but not quite, like a straight line.

The available data indicate that mice with pre-existing tetanus immunity represented by the intermediate range of about 0.004-0.128 u/ml plasma are exceedingly sensitive to booster stimulus. As a matter of fact, the hyper-reactivity of these mice was such that a mere threshold

booster dose of 0.005 Lf gave rise to a geometrical mean titer increase of 11-16 times, the extent of which was caught up by those mice with extremes of higher or lower residual immunity level only when a booster dose 10-fold stronger was used in the latter groups (Fig 1). Apparently due to the hyper-responsiveness of the former groups of mice to a threshold booster stimulus to yield a mean titer increase which was nearly maximal, further increase in titer resulting from dose augmentation was limited in the majority of cases, fallen short of the hypothetical titer increment value of 3.2-fold which was expected from the dose-response formula of Smith and St. John-Brooks (10). In contrast, a secondary response pattern contrary to that mentioned above appears to prevail in those mice with extremes of relatively high or low residual immunity. Here a barely detectable feeble response followed the threshold booster dose of 0.005 Lf. Different mechanisms seem to operate in these two categories of mice to account for such immunological unresponsiveness in face of a weak booster stimulus. The mice with low residual immunity represented by <0.001-0.002 u/ml plasma were likely in a state of minimal sensitization which was insusceptible to a threshold booster stimulus, while the relatively high circulating antitoxin concentration in those mice with 0.256-1.024 u/ml plasma rendered the minute strength of 0.005 Lf antigenically inactive by neutralization (11). In both cases however, once the immunological inertia, so to speak, was broken through by the threshold booster mass of antigen, further enhancement in booster dose resulted in the majority of cases, in a mean titer increase far beyond the square root of fold-increase in dose as is expected (2, 10).

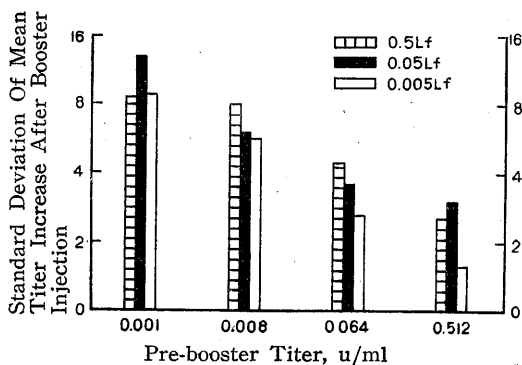


Fig 2. Correlation between standard deviation of mean titer increase and intensity of pre-existing immunity in secondary tetanus immunization in mice.

Another interesting finding is demonstrated in connection with the standard deviation of the mean titer increase after secondary tetanus immunization in mice. As shown in Table II and Fig 2, the greatest standard deviation invariably occurs in the groups of mice with the lowest pre-booster titer, decreasing progressively as the latter rises.

DISCUSSION

The present study leaves no doubt that the response lines of secondary tetanus immunization in mice are parabolic, in contrast to the straight response lines in men (1, 3). As pointed out elsewhere in the test, it was only when a big dose of 0.5 Lf was used in the mice that the response parabola approached the looks of a straight line—a result of an extraordinarily big dose indeed, since this would correspond to a fantastic dose of 500 Lf for men weighing 24–29 kg. It is admittedly difficult to compare critically the response patterns of secondary immunization between men and mice, since the human population always suffers from heterogeneity and the data on booster immunization in men are usually deficient in cases with exceedingly low prebooster titer, the latter due to the usual practice that they are immunized with a full course instead of an intentionally graded scale of primary doses. Nevertheless, the fact remains that the relationship in men between the log pre-booster titer and the log titer increase after booster injection is generally rectilinear (1–6), in distinct contrast to the parabolic response lines in mice as shown in the present study.

Taking 1/13 of the body weight as the criterion for calculating the total blood volume (12), the amount of individual pre-booster blood sample collected in this study represents about 1/3–1/5 of the total blood of mouse. This is certainly a tremendous amount of blood to be taken from an animal, but the balance of evidences are against the contention that the basic property of the response curves was determined by the process of excessive bleeding. 1) After bleeding, fairly long resting period of 8–10 days intervened before the secondary immunization was started. Not a single animal succumbed to the process of bleeding, and the generally healthy appearance of the animals suggested that they tolerated the bleeding well. 2) The response curves appear to be dose-dependent, *i.e.* different patterns can best be interpreted as an outcome

of the graded doses instead of the common cause of bleeding. 3) Repeated partial depletion of the circulating antibody, by withdrawing plasma, has been shown to have little effect on antibody formation (13). Although the evidences are in favor of the view that the response patterns described are characteristic in this species, which is known to be a poor antitoxin producer, it remains to be seen to what extent the response pattern would be modified if altered experimental conditions are used, such as the addition of adjuvant, the use of complex cellular antigen, the use of another mouse strain with different immunizability, *etc.*

Depending upon the level of residual immunity which is expressed by the concentration of circulating antitoxin, mice are in different states of reactivity to a given strength of booster tetanus stimulus. Those with an intermediate range of preexisting titers around 0.004–0.128 u/ml plasma are exceedingly sensitive to a threshold booster dose, so much so that further response to dose increment is limited. In contrast, those with initial titers either higher or lower than these limits are indifferent to a threshold stimulus, and yet subsequent titer increase in response to dose increment is exaggerative. These observations confirm our earlier finding that the immune status of the host, besides other factors, plays an important role in determining the dose-response relationship in secondary immunization (1). In the present study the booster doses of 0.005, 0.05 and 0.5 Lf induced a gross geometrical mean titer increase of 6.1-, 21.0- and 44.2-fold respectively, resulting in a titer gain ratio of 3.4 between the former two groups and 2.1 between the latter two. The former ratio of 3.4 agrees well with the hypothetical figure of 3.2 calculated after Steven's slope value of 0.5 of protein antigen (10), but the latter ratio of 2.1 falls short. Indeed, the possible existence of a ceiling phenomenon (2) in 0.5 Lf group can not be excluded to account for its relatively small mean titer gain of 44.2-fold, hence the smaller ratio of 2.1 between the groups of 0.05 and 0.5 Lf. Nevertheless, one can not emphasize the infallibility of applying a simple mathematical formula for interpretation of a complex biological phenomenon, particularly in secondary immune response where complicated response potentialities exist as shown in this study.

The current method of assaying tetanus toxoid in mice by primary immunization is liable to give faulty results, because the steepness of the dose-response slope obtained is easily affected by many variables such as the purity of toxoid

(14), the presence of adjuvant (15), the surrounding temperature (16), and the strain of mouse (17). A new approach of assaying tetanus toxoid in minimally sensitized mice has been tried in this laboratory, based on the observation mentioned above that mice with pre-existing titer around 0.001 u/ml plasma are exceedingly sensitive to the variation in booster dose. The result of preliminary evaluation of this method is encouraging. In 2 of the 3 lots of toxoid tested the new method was repeatedly capable of giving titration figures agreeable with those obtained by guinea pig method in another laboratory (Scheibel, I., personal communication). The results will be given in the next paper which is in preparation.

Inversional relationship between the level of pre-existing antibody titer and the size of standard deviation of mean titer-increase after specific antigenic stimulus was shown in this study. This represents a general immunological principle perhaps, since similar phenomenon occurs in men too (18). Generally speaking however, given the same sample size, such relationship could conceivably be demonstrated with more ease in experimental animals under laboratory control than in human-beings at large, because the heterogeneity of the human population might blur a true immunological picture. In situations where the question arises as to how many animals are required in a particular experiment (19), the immunological background of the animals should also be taken into account besides purely statistical considerations, especially when the initial titers encountered are low or non-existent, therefore the response variations are likely going to be large.

SUMMARY

The relationship between log titer increase and log pre-vaccination titer in secondary tetanus immunization in mice was studied in connection with the size of the immunizing dose. In this species, secondary immunization resulted in response parabolas the steepness of which decreased steadily as the intensity of booster dose increased. The standard deviation of mean titer increase was in inversion to the level of pre-booster immunity. The significance of these findings was discussed.

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REFERENCES

1. CHEN, B. L., C. T. CHOU, C. T. HUANG, Y. T. WANG, H. H. KO, W. C. HUANG, and C. F. LING. 1957. Studies on diphtheria-pertussis-tetanus combined immunization in children. III. Immune responses after the booster vaccination. *J. Immunol.* **79**: 393-400.
2. EDSALL, G., G. J. BANTON and R. E. WHEELER. 1951. The antigenicity of single, graded doses of purified diphtheria toxoid in man. *Amer. J. Hyg.* **53**: 283-295.
3. WISHART, F. O., and L. K. JACKSON. 1948. Tetanus toxoid-the recall dose. *Canad. J. Pub. Health.* **39**: 181-186.
4. MURRAY, E. S., A. OFSTROCK, and J. C. SNYDER. 1952. Antibody response of human subjects to epidemic typhus vaccine three to eight years after previous immunization. *J. Immunol.* **68**: 207-218.
5. HIRST, G. K., E. R. RICKARD, L. WHITMAN, and E. L. HORSFALL. 1942. Antibody response of human beings following vaccination with influenza viruses. *J. Exp. Med.* **75**: 495-511.
6. WOLF, A. M., H. J. SHAUGHNESSY, R. E. CHURCH, A. MILZER, M. JANOTA, F. OP-PDNHEIMER, R. A. MORRISSEY, H. NAFTULIN, J. W. CHAPMAN, and S. O. LEVINSON. 1956. Immunogenicity, in children, of ultraviolet-treated poliomyelitis vaccine. *J. Amer. Med. Assoc.* **161**: 775-781.
7. KASSEL, R., and S. LEVITAN. 1953. A jugular technique for the repeated bleeding of small animals. *Science* **118**: 563-564.
8. CHEN, B. L., C. T. CHOU, C. T. HUANG, Y. T. WANG, H. H. KO, and W. C. HUANG. 1956. Studies on diphtheria-pertussis-tetanus combined immunization in children. I. Heterologous interference of pertussis agglutinin and tetanus antitoxin response by pre-existing latent diphtheria immunity. *J. Immunol.* **77**: 144-155.
9. CHEN, B. L. 1959. A method for measuring low-titred tetanus antitoxin in small sample. (In Chinese with English summary). *Chin. Med J.* **6**: 7-10.
10. STEVENS, K. M. 1956. Some considerations of the antigen dose-antibody response relationship. *J. Immunol.* **76**: 187-191.
11. DIXON, F. J., S. C. BUKANTX, G. J. DAMMIN, and D. W. TALMAGE. 1953. p. 170-182. In A. M. Pappenheimer, Jr. (ed.) *The nature and significance of the antibody response*. Columbia University Press, New York.

12. REICHERT, E. T., and A. P. BROWN. 1909. *The crystallography of the hemoglobins*. Carnegie Inst., Washington.
13. SMOLENS, J., J. STOKES, E. MCGEE, and V. HUNTER. 1956. Feasibility and safety of frequent plasmapheresis of the same human donors. *Proc. Soc. Exptl. Biol. Med.* **91**: 611-614.
14. MURATA, R., E. WADA, A. YAMAMOTO, and K. KUBOTA. 1960. Studies on the quantitative measurement of tetanus toxoids, II. The international unit of tetanus toxoid and its error determined by mice method. (In Japanese). *Jap. J. Bact.* **15**: 866-870.
15. GREENBERG, L. 1953. International standard for tetanus toxoid. *Bull. Wld Hlth. Org.* **9**: 837-842.
16. WADA, E., A. YAMAMOTO, S. HIROSE, M. TAKAO, Y. OHTA, S. YAMAMOTO, S. MIYASAKE, and M. KOBAYASHI. 1959. Studies on the quantitative measurement of tetanus toxoid. I. On the immunizability of various mice strain against tetanus toxoids. (In Japanese). *Jap. J. Bact.* **14**: 546-551.
17. IPSEN, J. JR. 1954. Inherent immunizability to tetanus toxoid, based on studies in pure inbred mice. *J. Immunol.* **72**: 243-247.
18. SCHEIBEL, I., and S. TULINIUS. 1961. Simultaneous tetanus immunization and reinforcement of diphtheria immunity in adults, with special reference to the interference phenomenon. *Acta Path. et Microbiol. Scand.* **52**: 227-240.
19. DEARMON, I. A., JR., and R. E. LINCOLN. 1959. Number of animals required in the bioassay of pathogens. *J. Bact.* **78**: 651-657.